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Expression of the *Otx2* homeobox gene in the developing mammalian brain: embryonic and adult expression in the pineal gland

Martin F. Rath,*,† Estela Muñoz,†,[1] Surajit Ganguly,† Fabrice Morin,† Qiong Shi,† David C. Klein† and Morten Møller*

**Institute of Medical Anatomy, Panum Institute, University of Copenhagen, Copenhagen, Denmark*

†*Section on Neuroendocrinology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA*

Abstract

Otx2 is a vertebrate homeobox gene, which has been found to be essential for the development of rostral brain regions and appears to play a role in the development of retinal photoreceptor cells and pinealocytes. In this study, the temporal expression pattern of *Otx2* was revealed in the rat brain, with special emphasis on the pineal gland throughout late embryonic and postnatal stages. Widespread high expression of *Otx2* in the embryonic brain becomes progressively restricted in the adult to the pineal gland. *Crx* (*cone–rod homeobox*), a downstream target gene of Otx2, showed a pineal expression pattern similar to that of *Otx2*, although there was a distinct lag in time of onset. Otx2 protein was identified in pineal extracts and found to be localized in pinealocytes. Total pineal Otx2 mRNA did not show day–night variation, nor was it influenced by removal of the sympathetic input, indicating that the level of Otx2 mRNA appears to be independent of the photoneural input to the gland. Our results are consistent with the view that pineal expression of *Otx2* is required for development and we hypothesize that it plays a role in the adult in controlling the expression of the cluster of genes associated with phototransduction and melatonin synthesis.

Keywords: brain development, cone–rod homeobox, homeobox, *Otx2*, pineal gland.

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The *Otx2* homeobox gene is a vertebrate orthologue of the *Drosophila orthodenticle* gene (Finkelstein *et al*. 1990; Simeone *et al*. 1992, 1993); members of this orthology group play a fundamental role in development of photoreceptors [*Otx2* and *Crx* (*cone–rod homeobox*)] and rostral brain regions (*Otx1* and *Otx2*) (reviewed by Simeone *et al*. 2002; Arendt 2003). In the mouse embryo, *Otx2* is expressed in the prosencephalon and mesencephalon (Simeone *et al*. 1992), and knockout studies have shown that *Otx2* is essential for development of these brain regions (Acampora *et al*. 1995; Matsuo *et al*. 1995; Ang *et al*. 1996). *Otx2* also appears to play a role in development and function of the retina, in which the gene is expressed at both prenatal and postnatal stages (Bovolenta *et al*. 1997; Baas *et al*. 2000; Martinez-Morales *et al*. 2001; Viczian *et al*. 2003; Sakami *et al*. 2005). Microinjection experiments on *Xenopus* embryos indicate a role in eye field formation (Zuber *et al*. 2003). In addition, transfection experiments in mammalian systems suggest that *Otx2* is involved in differentiation of photoreceptor cells (Nishida *et al*. 2003; Akagi *et al*. 2004). *In vivo* evidence for the specific involvement of *Otx2* in the development of retinal photoreceptor cells and pinealocytes

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Address correspondence and reprint requests to Martin Fredensborg Rath, Institute of Medical Anatomy, Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen, Denmark. E-mail: m.rath@mai.ku.dk

[1]Estela Muñoz is a member of the National Council of Research, Science and Technology (CONICET), Argentina.

Abbreviations used: Aq, cerebral aqueduct; BTP, basal telencephalic plate; Ce, cerebellum; CP, cortical plate; Crx, cone–rod homeobox; E, embryonic day; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Ha, habenula; Hi, hippocampus; Hy, hypothalamus; Me, mesencephalon; MO, medulla oblongata; NC, neocortex; P, postnatal day; PBS, phosphate-buffered saline; Pi, pineal gland; Po, pons; SC, superior colliculus; SCGx, superior cervical ganglionectomy; Te, tectum; Th, thalamus; 3V, third ventricle; 4V, fourth ventricle; ZT, Zeitgeber time.

comes from a study on an *Otx2* conditional knockout mouse, which exhibits a lack of both cell types (Nishida *et al*. 2003).

Pinealocytes and retinal photoreceptor cells appear to have evolved from a common ancestral photoreceptor, as evidenced by ultrastructural similarities (Collin 1971) and expression in both tissues of many members of a set of genes dedicated to phototransduction and melatonin synthesis (reviewed by Klein 2004). The overlapping pattern of gene expression in these tissues seems to reflect control by similar mechanisms involving closely related homeobox genes, members of the Otx/Crx family (Chen *et al*. 1997; Furukawa *et al*. 1997, 1999; Li *et al*. 1998; Bernard *et al*. 2001; Gamse *et al*. 2002; Wang *et al*. 2002; Appelbaum *et al*. 2004, 2005). Indirect molecular evidence of a function of *Otx2* in both the retina and the pineal gland comes from *trans*-activation studies showing that Otx2 activates transcription of *Crx* (Nishida *et al*. 2003), which is strongly expressed in retinal photoreceptors and pinealocytes (Chen *et al*. 1997; Furukawa *et al*. 1997). *Crx* is known to play a role in transcriptional regulation of the last two enzymes in melatonin synthesis, arylalkylamine *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase (Li *et al*. 1998; Furukawa *et al*. 1999; Bernard *et al*. 2001).

The above observations are consistent with the view that *Otx2* plays two roles in the pinealocyte, one in determining cell fate and the second in maintaining phenotype. However, direct evidence that Otx2 mRNA and protein occur in the pineal gland is lacking, and such evidence is essential if we are to conclude that *Oxt2* is directly involved in controlling gene expression in this tissue. The results presented in this study establish that *Otx2* is expressed in the embryonic and adult pineal gland, consistent with the view that it plays a dual role in the pineal gland, determining cell fate and maintaining phenotype.

Materials and methods

Animals

Fetal and postnatal Sprague–Dawley rats for the developmental series were obtained from timed-pregnant animals (Charles River, Sulzfeld, Germany; Taconic Farms, Germantown, NY, USA) and killed during daytime at Zeitgeber time (ZT) 6–8. Brains were fixed by immersion in 4% paraformaldehyde for 2 days. Adult male Sprague–Dawley rats, weighing 200–300 g, were kept under a 14 h−10 h light–dark schedule. For Northern blotting, *in situ* hybridization and Western blotting, animals were killed by decapitation at ZT7 (day) and ZT19 (night) respectively (tissues unfixed). Bilateral superior cervical ganglionectomy (SCGx) (Møller *et al*. 1997) was performed 10 days before death. For immunohistochemistry, animals were perfusion-fixed in 4% paraformaldehyde during daytime. All experiments with animals were performed in accordance with the guidelines of *EU Directive 86/609/EEC* (approved by the Danish Council for Animal Experiments) and the *National Institutes of Health Guide for Care and Use of Laboratory Animals*.

In situ hybridization

Cryostat sections, 14 μm (developmental series) or 12 μm (adult animals) in thickness, were mounted on Superfrost Plus® slides. The sections were hybridized as described previously (Møller *et al*. 1997) with ^{35}S-labelled 38-mer antisense DNA probes corresponding to either bases 710–747 of the predicted rat Otx2 mRNA (XM_224009), 5′-CGAGCCAGCAT AGCCTTGACTATAACCTG-AAGCCTGAG-3′, or to bases 191–228 of rat Crx mRNA (NM_021855), 5′-GATCTTGAGAGCAACCTCCTCACGTGCA-TACACATCCG-3′. Sections were counterstained with cresyl violet.

Images of the sections on radiographs were transferred to a computer and quantified by using Image 1.42 software (Wayne Rasband, NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/nih-image). Optical densities were converted to dpm/mg tissue by using simultaneously exposed ^{14}C-standards calibrated by comparison with ^{35}S brain-paste standards. In the developmental series, the pineal signal was quantified on 2–6 sections from three animals at each time point. In other brain areas, which owing to their size were not present in all sections, the signal was quantified on 2–6 sections from 1–3 animals; therefore, *n* is not given in the figure legend. In adult animals, the pineal signal of four sections from five animals in each experimental group was quantified. The mean for each animal was calculated and the group mean ± SD was then determined. A two-tailed *t*-test was used for comparing means of pineal dpm/mg tissue in sections from animals killed during the day versus at night. A p-value of < 0.05 was considered to represent statistical significance.

Northern blot analysis

Total RNA was prepared from frozen tissues using TriZOL® (Invitrogen, Carlsbad, CA, USA). For the developmental series, pineal RNA was obtained from pools of 20 [embryonic day (E)18–E21], 10 [postnatal day (P)2–P12] or five (P19–P60) glands. The analysis was repeated in three independent experiments using different pools. Some 8 μg total RNA was loaded per lane in a 1.5% agarose/0.7 M formaldehyde gel. For analysis of adult animals, RNA was obtained from pools of tissue from five animals at each time point. The analysis was repeated in two independent experiments using different pools. Some 6 μg total RNA was loaded per lane in a 1.0% agarose/0.7 M formaldehyde gel and separated by electrophoresis in a 1 × MOPS (50 mM) buffer (Quality Biological, Gaithersburg, MD, USA). Membrane transfer and hybridization were performed as described previously (Kim *et al*. 2005). Hybridization probes corresponding to bases 659–1033 of the predicted rat Otx2 mRNA (XM_224009) or to bases 288–597 of rat Crx mRNA (NM_021855) were obtained by PCR using rat pineal cDNA as template. Complementary DNA was synthesized from night pineal total RNA on Dynabeads® (Dynal, Brown Deer, WI, USA) using a combination of MasterAmp® Tth DNA polymerase (Epicentre, Madison, WI, USA) and Superscript II® reverse transcriptase (Invitrogen). The reaction was incubated at 40°C for 30 min, then at 70°C for 1 h.

The blots were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and analysed with ImageQuant™ software. Data were corrected for loading differences and expressed as mean ± SD. Transcript sizes were estimated by comparison with standard RNA markers (Roche, Indianapolis, IN, USA). For analysis of adult animals, the blot was stripped and rehybridized

with a 983-bp probe corresponding to residues 854–1836 of rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA (NM_017008).

Immunohistochemistry

Cryostat sections 14 μm thick were cut from three animals and mounted on Superfrost Plus® slides. Immunohistochemistry was performed as described previously (Mukda *et al*. 2005). Primary antibody, polyclonal goat anti-human Otx2 IgG (R & D Systems, Abingdon, UK), was diluted to 10 μg/mL; secondary antibody, biotinylated donkey anti-goat IgG (Jackson, Soham, UK), was diluted 1 : 500. Chromogenic development was done by incubation in Avidin-Biotin-peroxidase complex followed by diaminobenzidine (Mukda *et al*. 2005).

Western blot analysis

Samples were obtained from pools of fresh tissues from five animals at each time point. The analysis was repeated in two independent experiments using different pools. Samples were homogenized in 2 × Laemmli buffer containing 73% 155 mM Tris buffer (pH 8.3), 9% sodium dodecyl sulphate, 16 mM bromphenol blue, 18% glycerol and 10% 2-mercaptoethanol (0.1 g tissue/mL buffer). Samples were boiled and centrifuged at 13 000 *g* for 1 h at 4°C. Protein content of the supernatants was determined using a RC DC Protein Assay (Bio-Rad, Hercules, CA, USA). Some 50 μg protein per lane was run in a NuPAGE® Bis-Tris 12% gel (Invitrogen) and transferred to a nitrocellulose membrane by use of the XCell® Surelock Mini-Cell system (Invitrogen). The membrane was blocked in blocking solution (Amersham, Hillerød, Denmark). The membrane was incubated in 6 μg/mL primary antibody (as for immunohistochemistry) diluted in blocking solution for 1 h and washed in phosphate-buffered saline (PBS). The membrane was subsequently incubated in biotinylated secondary antibody (as for immunohistochemistry) diluted 1 : 500 in blocking solution for 1 h and washed in PBS. The membrane was incubated in ABC Vectastain (Vector, Burlingame, CA, USA) diluted 1 : 100 in blocking solution and subsequently washed in PBS and 0.05 M Tris (pH 7.6). Chromogenic development was done by incubating the membrane for 1 min in 1.4 mM diaminobenzidine (Sigma) and 0.01% H_2O_2 in 0.05 M Tris (pH 7.6); the reaction was stopped by washing in deionized water. Protein size was estimated by comparison with standard protein molecular weight markers (Amersham).

Molecular cloning and sequence analysis

Two primers, 5′-ATGATGTCTTATCTAAAGCA-3′ and 5′-TCACAAAACCTGGAATTTCC-3′, corresponding to the extreme ends of the predicted 870-bp rat *Otx2* open reading frame (XM_224009), were used for PCR with rat pineal cDNA as template; cDNA was prepared as described for Northern blotting. The PCR product was cloned using the pGEM®-T Easy Vector System (Promega, Madison, WI, USA) and transformed into MAX Efficiency® DH5α cells (Invitrogen). Transformed cells were selected on LB plates containing ampicillin (100 μg/mL) and positive clones were screened by PCR using insert-specific primers corresponding to the extreme ends of the rat *Otx2* open reading frame (see above). Plasmids from positive clones were isolated using Wizard® Plus SV Minipreps (Promega) and sequenced commercially in both directions by Veritas (Rockville, MD, USA) using vector-specific T7 and SP6 primers and the Sanger method.

Results

Abundant *Otx2* expression of the developing brain becomes progressively restricted to the pineal gland

To determine the ontogenetic expression pattern of *Otx2* in the pineal gland, *in situ* hybridization was performed on sagittal sections through the brains of animals killed in a developmental series ranging from E16 to P30 (Fig. 1). The rat pineal gland develops as a dorsal evagination from the most caudal part of the diencephalic roof and appears as a small tubular evagination at E16 (Calvo and Boya 1981). Here, we found that *Otx2* was expressed in the pineal gland at E16; however, stronger expression was seen in the mesencephalic tectum, in which all layers were labelled. *Otx2* expression was also detected in the choroid plexus, the subventricular areas of the thalamus and the basal telencephalic plate. After E16, *Otx2* was abundantly expressed in the pineal at all stages investigated. In the tectum, at E18, the expression was confined to the superficial and deep layers, and from E19 expression was seen in the superior colliculus. *Otx2* expression was also detected in the habenula. In the cerebellum, *Otx2* expression was observed in the most caudal superficial areas from E16; expression was seen in the external germinal layer and granular layer from P2 to P18. At P30, the cerebellar expression was weak and confined only to the granular layer.

Densitometric quantification of Otx2 mRNA revealed an expression pattern similar to that observed above (Fig. 2a), with a high level of pineal expression throughout development, whereas in other brain regions the intensity of the signal declined markedly or disappeared.

Expression of *Otx2* in the pineal gland was confirmed by Northern blot analysis in a developmental series, which revealed the presence of a strong band corresponding to a transcript of approximately 2.5 kb at all stages examined from E18 to P60 (Fig. 3a). This transcript size is in accord with previous studies on the mouse (Simeone *et al*. 1993; Courtois *et al*. 2003). Minor bands (2 kb and 4 kb) were also observed.

Crx expression in the developing pineal gland succeeds the expression of *Otx2*

The sustained high level of expression of *Otx2* in the pineal gland prompted us to compare its developmental expression profile with that of *Crx*, a downstream target of Otx2 (Nishida *et al*. 2003) (Fig. 1). At E16 and E17, Crx mRNA was not detected in the pineal gland or in other regions of the brain present on median sections. From E18 onwards, Crx mRNA was detected in the pineal gland. In marked contrast

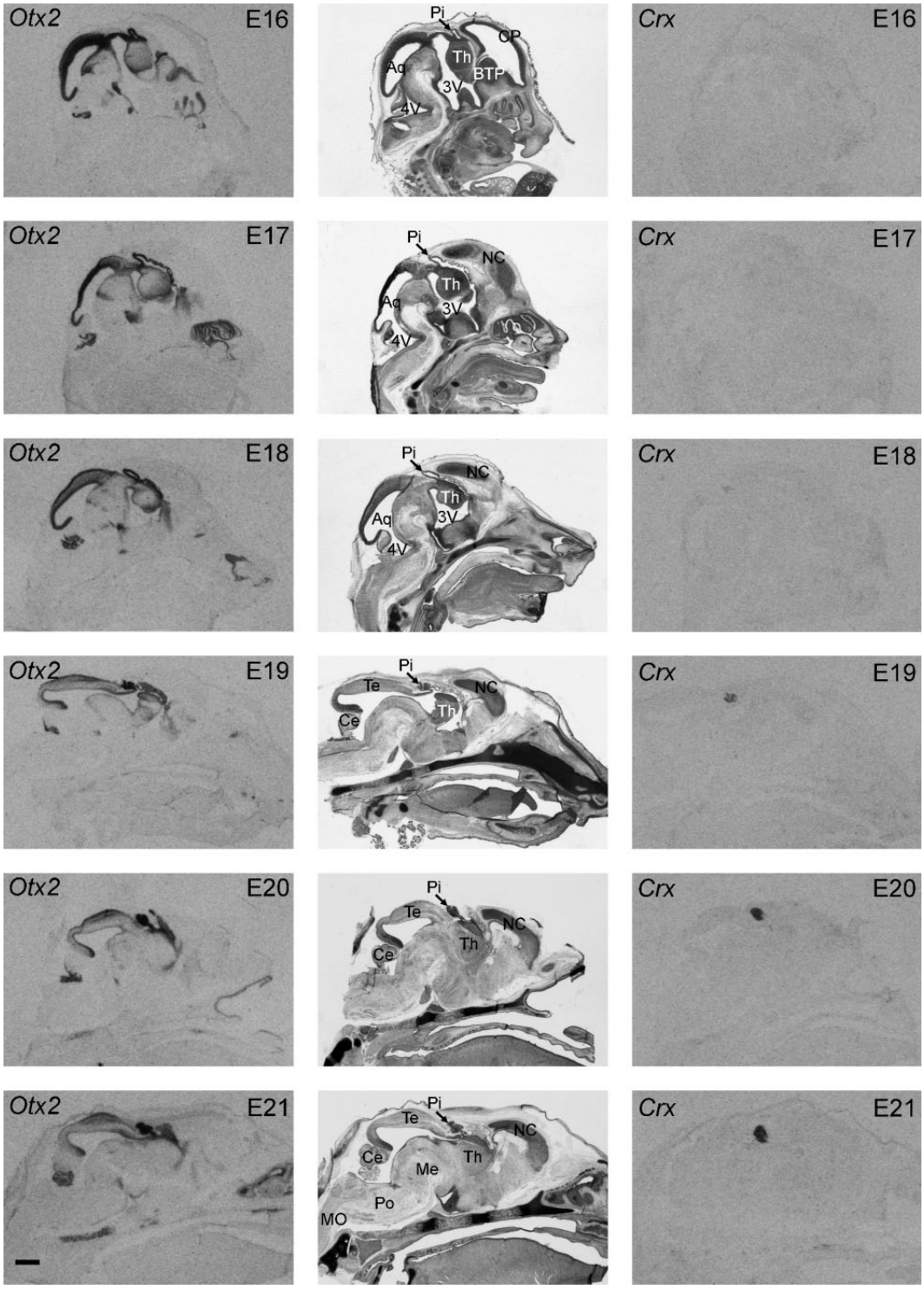

Fig. 1 Developmental *in situ* hybridization series for detection of *Otx2* and *Crx* expression in the rat brain. Left column: radiographs of *in situ* hybridization for detection of Otx2 mRNA on median sections through the brains of embryonic and postnatal rats. The developmental stages are indicated in the upper right corner of each photomicrograph (E16–P30). Middle column: counterstained sections corresponding to the radiographs. Right column: radiographs of *in situ* hybridization for detection of Crx mRNA on median sections through the brains of embryonic and postnatal rats. 3V, third ventricle; 4V, fourth ventricle; Aq, cerebral aqueduct; BTP, basal telencephalic plate; Ce, cerebellum; CP, cortical plate; Ha, habenula; Hi, hippocampus; Hy, hypothalamus; Me, mesencephalon; MO, medulla oblongata; NC, neocortex; Pi, pineal gland; Po, pons; SC, superior colliculus; Te, tectum; Th, thalamus. Scale bar 1 mm.

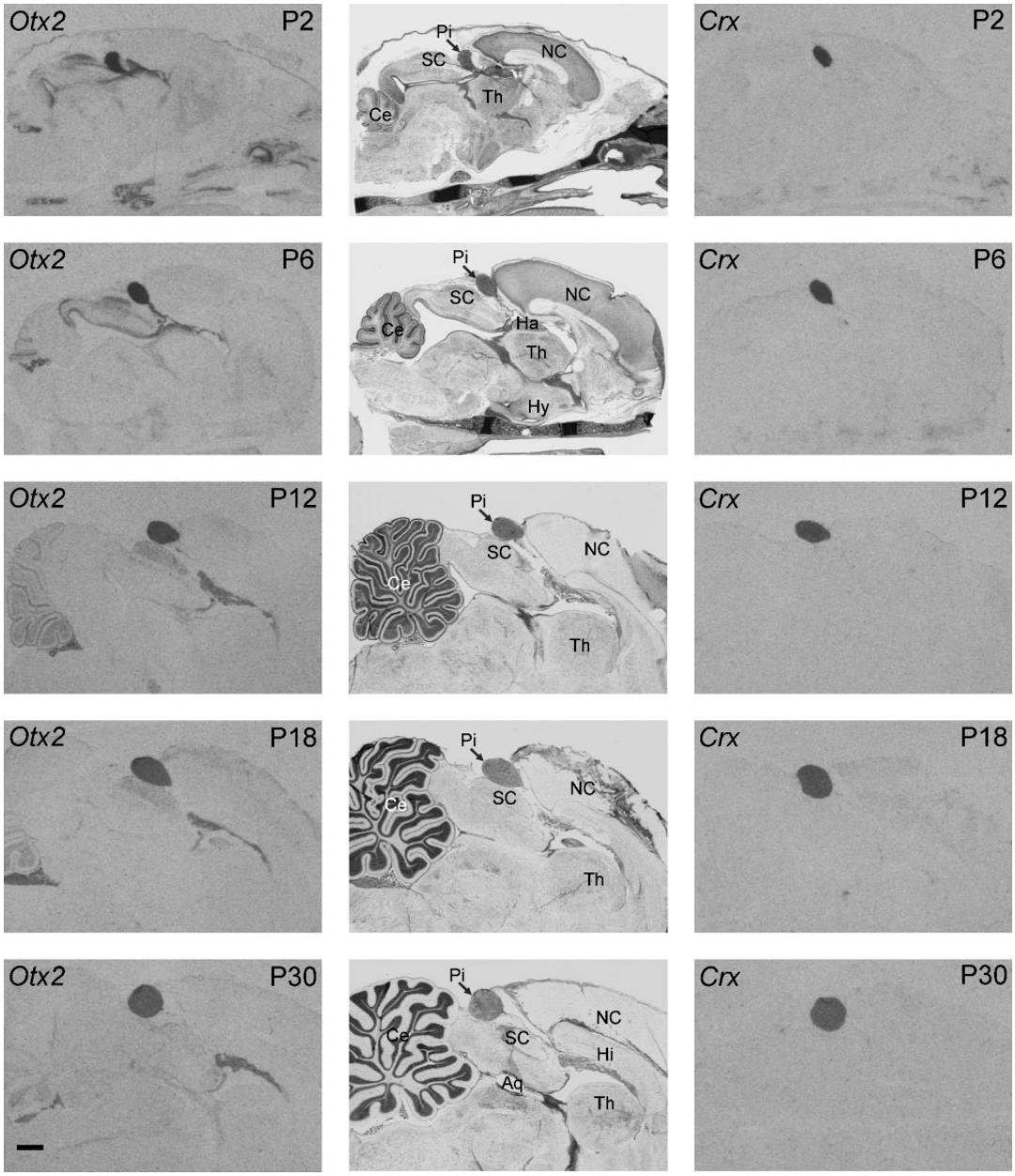

Fig. 1 Continued.

to the tissue distribution pattern of *Otx2*, the expression of *Crx* was strictly confined to the pineal gland at all stages examined. *Crx* expression in the developing pineal gland was confirmed by Northern blot analysis (Fig. 3b).

Densitometric analysis of the pineal Otx2 mRNA indicated that expression appeared to peak at E20 and be maintained thereafter, albeit at a somewhat reduced level (Fig. 2b). The expression of *Crx* in the gland was also found to increase from the first detectable level at E18 towards a peak just around birth from E21 to P2, before stabilizing at a lower level in the adult pineal gland. Comparison of the developmental patterns of pineal expression of *Otx2* and *Crx* revealed that the marked increase in *Crx* expression occurred 2 days later than that of *Otx2*.

Otx2 mRNA levels in the adult pineal gland and retina are similar

Examination of the temporal and spatial expression pattern of *Otx2* in the adult rat CNS by Northern blotting indicated that the pineal gland and retina had similar multiple band patterns (2.0, 2.5 and 4.0 kb) (Fig. 4). Weak *Otx2* expression was seen in the neocortex and cerebellum, but was undetectable in the spinal cord (data not shown). The 2.0-kb isoform was weakly expressed, especially in the retina. The 4-kb

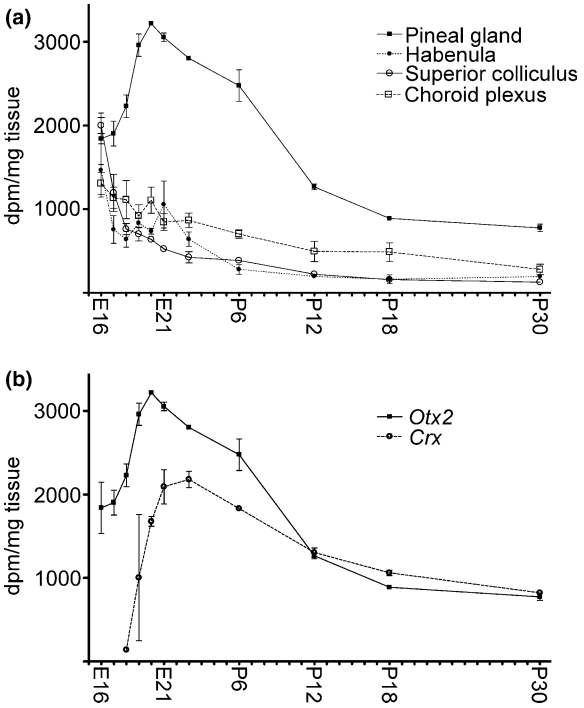

Fig. 2 Densitometric quantification of the signal of the *in situ* hybridization X-ray images of the developmental series. (a) Expression of *Otx2* in the pineal gland, habenula, superior colliculus (mesencephalic tectum at early stages) and choroid plexus of the fourth ventricle at developmental stages indicated. (b) Expression of *Otx2* and *Crx* in the pineal gland at developmental stages indicated Values are mean ± SD ($n = 3$).

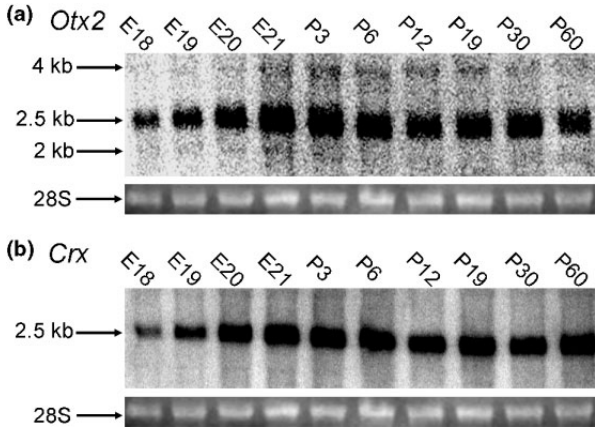

Fig. 3 *Otx2* and *Crx* are expressed in the developing pineal gland. Northern blot analysis of (a) pineal *Otx2* expression and (b) pineal *Crx* expression at the developmental stages indicated. The lower panel in each figure shows the 28S ribosomal RNA bands on the corresponding gel used as a loading control.

transcript exhibited a moderate diurnal rhythm in the pineal gland, with raised levels at night (night/day ratio: 2.1 ± 0.3).

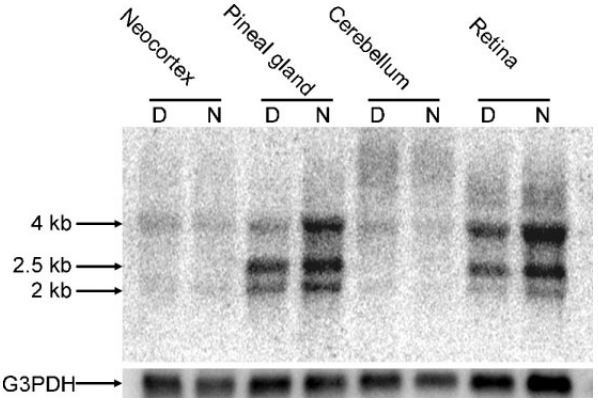

Fig. 4 Northern blot analysis of *Otx2* expression in tissues removed from adult animals killed at midday (D, ZT7) and midnight (N, ZT19), respectively. Arrows on the upper image indicate the transcript lengths. The lower panel displays the same blot hybridized with a probe detecting G3PDH mRNA as a loading control.

In situ hybridization of sagittal brain sections from animals killed at midday and midnight revealed a strong signal in the superficial pineal gland at both time points (Fig. 5), in accordance with the Northern blot data. Densitometric quantification of *Otx2* expression in the superficial pineal gland revealed no significant day–night variation (unpaired *t*-test, $p = 0.06 > 0.05$), in contrast to the results of Northern blot analysis. The sequence of the Otx2 mRNA corresponding to the *in situ* probe is contained within that of the probe used for Northern blotting; accordingly, the diurnal rhythm of the 4-kb transcript may be masked by the contribution of the shorter isoforms to the total pool of Otx2 transcripts.

To determine whether the expression of *Otx2* is controlled by the sympathetic neural input that controls pineal function (Klein *et al*. 1971; Sugden and Klein 1983; Klein 1985) animals were subjected to bilateral SCGx (Fig. 5). This blocks neural stimulation of the gland, but did not abolish *Otx2* expression, indicating that intact sympathetic innervation of the pineal gland is not required to maintain Otx2 mRNA levels in this tissue.

Otx2 protein is highly abundant in the pineal gland of the adult rat

The predicted sequence of the rat Otx2 protein (XP_2204009) has a molecular weight of 31.6 kDa. Western blot analysis revealed the presence of a single strong 30-kDa protein band in the pineal gland and the retina (Fig. 6), establishing that the Otx2 transcript is translated in these tissues. Otx2 protein was detected at very low levels in cerebellar extracts, but not in extracts of the neocortex or the spinal cord. Diurnal variations in expression levels of Otx2 in the pineal gland and retina were not observed.

The cellular localization of the Otx2 protein was investigated by immunohistochemical staining of brain sections

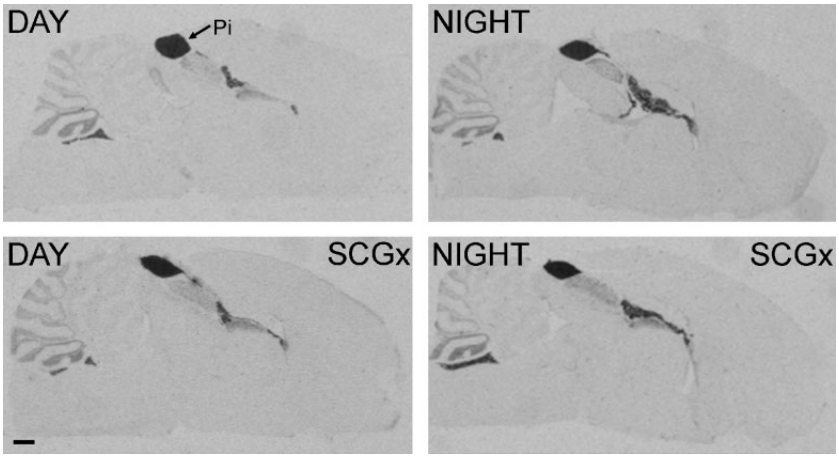

Fig. 5 *In situ* hybridization on median sections through brains of adult rats hybridized for detection of Otx2 mRNA. Upper panel: the section on the left is from an animal killed at midday (ZT7) and that on the right is from an animal killed at midnight (ZT19). A strong signal was noted in the pineal gland at both time points. Lower panel: sections from animals that had undergone SCGx, killed at midday (ZT7) and midnight (ZT19). Scale bar 1 mm.

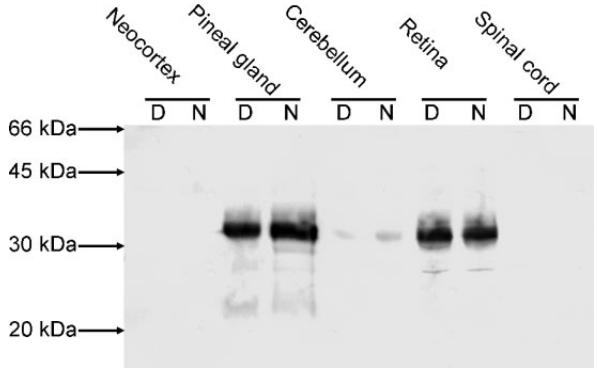

Fig. 6 Western blot analysis of expression of the Otx2 protein in parts of the CNS removed from adult animals killed at midday (D, ZT7) and midnight (N, ZT19). The predicted sequence of the rat Otx2 protein (XP_2204009) has a predicted molecular weight of 31.6 kDa. Arrows indicate molecular weights determined using standard molecular weight markers.

from animals killed during daytime. Strong labelling was detected in pinealocytes of both the superficial (Fig. 7a) and the deep pineal gland. The cytoplasm of the majority of cells in the pineal gland was immunoreactive for Otx2.

Otx2 immunoreactivity was also evident in the ependymal cells of the choroid plexus of all four ventricles. Furthermore, Otx2 immunoreactivity was detected in the superficial layers of the superior colliculus (Fig. 7b), the lateral geniculate nuclei with the highest density in the ventral subnucleus (Fig. 7c), the dorsal terminal nucleus of the accessory optic tract, the parafascicular thalamic nucleus, the arcuate nucleus, the median eminence, the periventricular area of the hypothalamus and the suprachiasmatic nucleus (Fig. 7d). In the suprachiasmatic nucleus, general staining of the whole nucleus was seen in addition to some intensively stained cells. In the cerebellum, the Purkinje cells exhibited moderate Otx2 immunoreactivity.

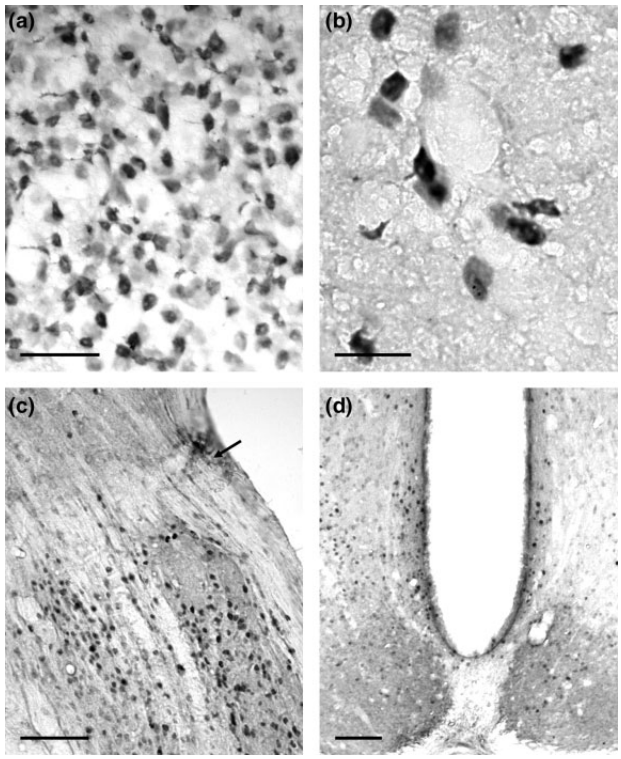

Fig. 7 Immunohistochemical visualization of the Otx2 protein in rat brain sections. (a) Sagittal section of the superficial pineal gland. Immunoreactivity was present in many pinealocytes. Scale bar 50 μm. (b) Coronal section of the superior colliculus. Immunoreactivity was noted in several neurones of the superficial layers. Scale bar 20 μm. (c) Coronal section of the lateral geniculate nucleus. The arrow indicates the intergeniculate leaflet separating the ventral lateral geniculate nucleus (lower part of image) with a high density of immunoreactive cells from the dorsal lateral geniculate nucleus (upper part of image). Scale bar 100 μm. (d) Coronal section through the suprachiasmatic area of the hypothalamus. The suprachiasmatic nucleus was clearly delineated from the surrounding parts of the hypothalamus. Scale bar 100 μm.

Molecular cloning and sequencing of the rat *Otx2* open reading frame

Clones containing the *Otx2* open reading frame were isolated from a pineal cDNA library and sequenced. This revealed the existence of two distinct rat *Otx2* open reading frames, with lengths of 870 and 894 bp, differing only by an in-frame 24-bp sequence. Alignment of the two *Otx2* sequences with the rat genomic sequence (NW_047453) indicated alternative splicing at the 5′-end of the predicted second exon as the mechanism generating two alternative open reading frames in the Otx2 mRNA. A similar organization of the *Otx2* open reading frame has been reported in the mouse (Courtois *et al*. 2003).

Discussion

The results presented here represent the first direct demonstration of *Otx2* expression and the presence of Otx2 protein in the pineal gland, and are consistent with the findings of previous work demonstrating that *Otx2* is required for pineal development (Nishida *et al*. 2003). These observations, together with reports of *Otx2* expression in the retina at both prenatal and postnatal stages (Bovolenta *et al*. 1997; Baas *et al*. 2000; Martinez-Morales *et al*. 2001; Nishida *et al*. 2003; Viczian *et al*. 2003; Sakami *et al*. 2005) and recent molecular evidence (Nishida *et al*. 2003), are in accord with the view that *Otx2* plays a role in both the retina and the pineal gland.

The expression pattern of the *Otx2* homeobox gene in the early developing CNS (Simeone *et al*. 1992; Simeone *et al*. 1993; Mallamaci *et al*. 1996) and its role in patterning of the rostral part of the brain (Matsuo *et al*. 1995; Acampora *et al*. 1995; Ang *et al*. 1996) seem well established. Our finding that the *Otx2* gene is highly expressed in the pineal gland throughout pineal development into adulthood is of further interest because it argues for a role of the protein in maintaining pinealocyte phenotype in addition to determining cell fate. In this regard, the cytoplasmic localization of Otx2 protein in pinealocytes is in accord with the subcellular distribution of Otx2 protein in differentiated rod photoreceptors, in which an important role for sequestering of Otx2 to the cytoplasm in maintaining post-mitotic cell fate has been proposed (Baas *et al*. 2000). In contrast, the observation that *Otx2* expression in other non-pineal areas of the brain markedly decreases during development indicates that *Otx2* has a broad primary role in determination of fate in these areas. However, the presence of a limited number of additional Otx2-immunoreactive cells in the CNS provides reason to suspect that Otx2 also plays a role in maintaining phenotype in these cells, as in pinealocytes. This view is supported by recent studies of conditional knockout mice indicating a role for *Otx2* in regulation of neuronal subtype identity (Puelles *et al*. 2004; Vernay *et al*. 2005).

It is of interest that Otx2-immunoreactive neurones are present in nuclei of central pathways of the visual system, including the lateral geniculate nuclei, the dorsal terminal nucleus of the accessory optic tract, the superior colliculus and also the suprachiasmatic nucleus. We hypothesize that the presence of Otx2 in neuron of the optic pathways might reflect intercellular transport of Otx2 protein between synapsing neurones and subsequent autoinduction of gene expression, as described previously (Prochiantz and Joliot 2003). Furthermore, Otx2-positive structures include the cerebellar cortex and the choroid plexus, both of which were shown to contain transcript by *in situ* hybridization. These data are generally in accordance with previous *in situ* studies on postnatal rat (Frantz *et al*. 1994; Nothias *et al*. 1998). The results indicate that, even though the *Otx2* gene is highly expressed in the pineal gland and in the retina, its expression is not strictly confined to these tissues. This implies that tissue-specific expression of Otx2 downstream targets, e.g. *Crx* (this study; Chen *et al*. 1997; Nishida *et al*. 2003) and also the interphotoreceptor retinoid-binding protein gene (van Veen *et al*. 1986a; Bobola *et al*. 1998) in pinealocytes and retinal photoreceptors, is either dependent on a large amount of Otx2 for transcriptional activation or the influence of one or more transcription modulators, or a combination.

Crx has previously been shown to be expressed in the pineal gland (Chen *et al*. 1997; Li *et al*. 1998; Wang *et al*. 2002). The ontogenetic investigation of *Crx* in this study confirms this and also establishes that the *Crx* gene is expressed in the embryonic pineal gland starting at E18. Comparison of the developmental expression patterns of *Crx* and *Otx2* revealed a general similarity in that both are strongly expressed in the developing and adult pineal gland. However, a marked temporal difference in the onset and increase in expression was observed; *Otx2* expression started at least 2 days before that of *Crx*, providing further reason to support the view that Otx2 is a transcriptional activator of *Crx* (Nishida *et al*. 2003).

Immunocytochemical analysis revealed that the Otx2 protein is present in most, but not all pinealocytes, as also noted in immunocytochemical studies of Crx in mouse pineal gland (Wang *et al*. 2002). If *Crx* expression is dependent on transcriptional activation by Otx2 (Nishida *et al*. 2003), these genes should be active in the same subset of cells; therefore, one can predict that future studies are likely to find that Otx2 and Crx are co-localized in pinealocytes.

Our results indicate that the *Otx2* gene is expressed in the adult rat pineal gland and retina to a similar degree. These data add to the growing body of evidence that *Otx2* is expressed in the mature retina (Bovolenta *et al*. 1997; Baas *et al*. 2000; Martinez-Morales *et al*. 2001; Viczian *et al*. 2003; Sakami *et al*. 2005). The finding of similar levels of expression in both tissues argues for an important role of this transcription factor in both structures.

Our studies revealed multiple Otx2 transcripts. However, there is a distinct difference in the nature of this 'macro' heterogeneity, as compared to the 'micro' heterogeneity reported previously, based on studies on the mouse retina (Courtois *et al*. 2003). We found that there appears to be a large range in size of Otx2 transcripts in the rat (2.0–4.0 kb); this has not been seen in the mouse, in which Otx2 transcripts of similar size (~ 2.5 kb) have been documented (Courtois *et al*. 2003). Our comparison of the rat and mouse gene has failed to identify distinct differences that would explain the presence of 2- and 4-kb transcripts in the rat but not the mouse. The 4-kb band may reflect generation of transcripts containing a 1.8-kb intron that is otherwise spliced out. Inclusion of this intron would result in a transcript encoding a severely truncated protein (~ 10 kDa); such a product is unlikely to share biological activity with Otx2. The observed 'macro' heterogeneity may also reflect the use of cryptic polyadenylation sites.

The present study also provides evidence of 'micro' heterogeneity in the rat, because cloning and sequencing revealed two alternative *Otx2* open reading frames differing by a 24-bp sequence at the 5′-end of the second coding exon. Identical 24-bp in-frame segments encoding an octapeptide just N-terminal to the Otx2 homeodomain have been reported in mouse (Courtois *et al*. 2003) and human (NM_021728) Otx2 mRNAs; this appears to reflect a conserved mechanism for alternative splicing of the *Otx2* open reading frame in mammals. Western blot analysis revealed only one strong band of Otx2 protein; this is not surprising because the small difference in the mass of the protein resulting from the eight-residue difference in size is unlikely to be detected by the methods used.

An interesting finding in this study relates to the factors controlling pineal *Otx2* expression. As mentioned above, pineal function is controlled by a neural system, which terminates in sympathetic fibres from the superior cervical ganglion; noradrenaline, released from the sympathetic terminals, binds to adrenoceptors on the pinealocyte membrane activating a cyclic AMP second messenger system (Klein *et al*. 1997). Removal of the sympathetic input to the rat pineal has been shown to affect the expression of other pineal-specific genes (Stehle *et al*. 1993; Baler *et al*. 1996; Roseboom *et al*. 1996; Gaildrat *et al*. 2005; Kim *et al*. 2005). In contrast, total Otx2 mRNA abundance in the pineal gland was not markedly influenced by sympathetic denervation, indicating that the sympathetic input, which mediates photoneural control of the pineal gland, is not essential for maintaining *Otx2* expression. Rather, it appears that autonomous mechanisms are primarily responsible for expression of *Otx2*, and that this autonomous expression in turn contributes to maintenance of pinealocyte phenotype; the observation of a daily rhythm in the minor 4-kb transcript suggests that secondary mechanisms may modulate *Otx2* expression.

The abundant expression of the *Otx2* gene in both the pineal gland and the retina supports a common ancestral origin of these structures, as suggested previously from morphological studies (Collin 1971; Oksche 1971; Eakin 1973; Møller 1978, 1986) and molecular/biochemical data (Klein 2004; Somers and Klein 1984; Korf *et al*. 1985, 1992; Rodrigues *et al*. 1986; van Veen *et al*. 1986a, 1986b; Reig *et al*. 1990; Coon *et al*. 1995; Gauer and Craft 1996; Blackshaw and Snyder 1997). In this regard, Otx2 is a member of a group of Otx transcription factors expressed in the vertebrate retina and the pineal gland, which includes Crx and Otx5 (Chen *et al*. 1997; Gamse *et al*. 2002). In addition, our developmental data indicate placement of *Otx2* above *Crx* in the transcriptional cascade regulating similar gene expression, e.g. cellular fate, in both of these tissues.

In conclusion, the *Otx2* gene is expressed in several areas of the developing brain. During early ontogenesis, expression in most brain regions markedly decreases, whereas expression in the pineal gland and retina remains high, suggesting a transition from involvement in general morphogenesis in the CNS to a more narrowly defined role in these specific structures. Accordingly, *Otx2* appears to play a critical role in maintaining cell identity throughout life in both the retina and pineal gland.

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